Profound Perturbation – Immunopathological Mechanisms in Common Variable Immunodeficiency

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There are some enterprises in which a careful disorderliness is the true method.

H Melville

The pursuit of an exciting career choice is one of them. When I moved with my family to Oslo in 2003 with nothing but a scholarship pending the decision of the Research Council to show for, life did seem disordered. However, this move proved to be the true method of introduction to a field of work with many opportunities of which I am grateful to a number of people and institutions.

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Oslo, December 2009

TABLE OF CONTENTS

SELECTED ABBREVIATIONS

- APC Antigen presenting cell
- APRIL A proliferation inducing ligand
- BAFF B cell activating factor
- BAFF-R B cell activatin factor receptor
- BLyS B lymphocyte stimulator
- Btk Bruton's tyrosin kinase
- CD Cluster of differentiation
- CVID Common variable immunodeficiency
- DC Dendritic cell
- FoxP3 Forkhead helix P3
- HIV Human immunodeficiency virus
- ICOS Inducible costimulator
- IFN-γ Interferon gamma
- Ig Immunoglobulin
- IL Interleukin
- ITP Idiopathic thrombocytopenia
- MBL Mannose binding lectin
- MCP-1 Monocyte chemoattractant peptide 1
- MHC Major histocompatibility complex
- NK-cell Natural killer cell
- PAMP Pathogen-associated molecular pattern
- PBMC Peripheral blood monocytes
- PHA Phytohemagglutinin
- PRR Pathogen recognizing receptors
- suPAR Soluble urokinase plasminogen activator receptor
- TACI Transmembrane activator and calcium-modulating cyklophilin ligand interactor
- T_H T helper cell

- TLR Toll-like receptor
- TNFRSF13b Tumor necrosis factor receptor super family 13 b
- TNFRSF13c Tumor necrosis factor receptor super family 13 c
- TNF-α Tumor necrosis factor alpha
- T_{Reg} Regulatory T cell
- uPA Urokinase plasminogen activator
- uPAR Urokinase plasminogen activator receptor
- XLA X-linked agammaglobulinemia (Bruton's agammaglobulinemia)

LIST OF PAPERS

I. Fevang B, Mollnes TE, Holm AM, Ueland T, Heggelund L, Damås JK, Aukrust P, Frøland SS. Common variable immunodeficiency and the complement system; low mannose-binding lectin levels are associated with bronchiectasis. Clin Exp Immunol 2005 Dec;142(3):576-84.

II. Fevang B, Eugen-Olsen J, Yndestad A, Brosstad F, Beiske K, Aukrust P, Frøland SS. Enhanced levels of urokinase plasminogen activator and its soluble receptor in common variable immunodeficiency. Clin Immunol 2009. In press.

III. Fevang B, Yndestad A, Damås JK, Bjerkeli V, Ueland T, Holm AM, Beiske K, Aukrust P, Frøland SS. Chemokines and common variable immunodeficiency; possible contribution of the fractalkine system (CX3CL1/CX3CR1) to chronic inflammation. Clin Immunol 2009 130, 151-161.

IV. Fevang B, Yndestad A, Damås JK, Halvorsen B, Holm AM, Beiske K, Aukrust P, Frøland SS. Chemokines and common variable immunodeficiency; possible contribution of CCL19, CCL21 and CCR7 to immune dysregulation. Submitted.

V. Fevang B, Yndestad A, Sandberg WJ, Holm AM, Müller F, Aukrust P, Frøland SS. Low numbers of regulatory T cells in common variable immunodeficiency: association with chronic inflammation in vivo. Clin Exp Immunol 2007 Mar;147(3):521-5.

1. INTRODUCTION

The integrity of all organisms to injury is conserved through intricate mechanisms of response, but none more so than the immune system of the mammals. The complexity and potency of these mechanisms make them both difficult and intriguing to dissect, but more importantly, the study of the immune system can be of benefit both therapeutically and diagnostically in a range of human diseases. While much have been learnt by studying *how the immune system works* in both immune and non-immune diseases in humans and animals, the study of human immunodeficiencies gives an unique opportunity to study the consequences of *an immune system inept to work*. In this thesis I will present the work done by my colleagues and myself on innate, inflammatory and regulatory immune mechanisms in Common variable immunodeficiency (CVID), an immunodeficiency named more in keeping with the usual definition of "variable" than "common", but with manifestations highlighting important principles of our current understanding of the immune system. In this introduction I will first briefly outline the normal human immune response before exploring parts of particular interest to my thesis. Thereafter, I will give a brief overview over human immunodeficiencies before examining the particular features of CVID in depth.

1.1 THE IMMUNE RESPONSE

The definition of the immune response requires some consideration as it has evolved in later years. While a broad definition includes the reaction of the body to any substance foreign or interpreted as foreign (1), the most common understanding is a response involving both specific and unspecific responses by leukocytes and proteins (2). Some, however, prefer a more strict and traditional definition including only reactions depending on specific reactions to antigens and the development of immunological memory (3). The growing evidence of close cooperation of all cellular components involved in the reaction to foreign substances would support a broad definition. Furthermore, while immunology traditionally has dealt with the response to infectious agents like bacteria, virus and parasites, it is clear that many of the same responses also apply to the human body's reaction to toxins, malignant cells and even innocuous self-antigens causing auto-immune disease (4). Here however, I will sketch

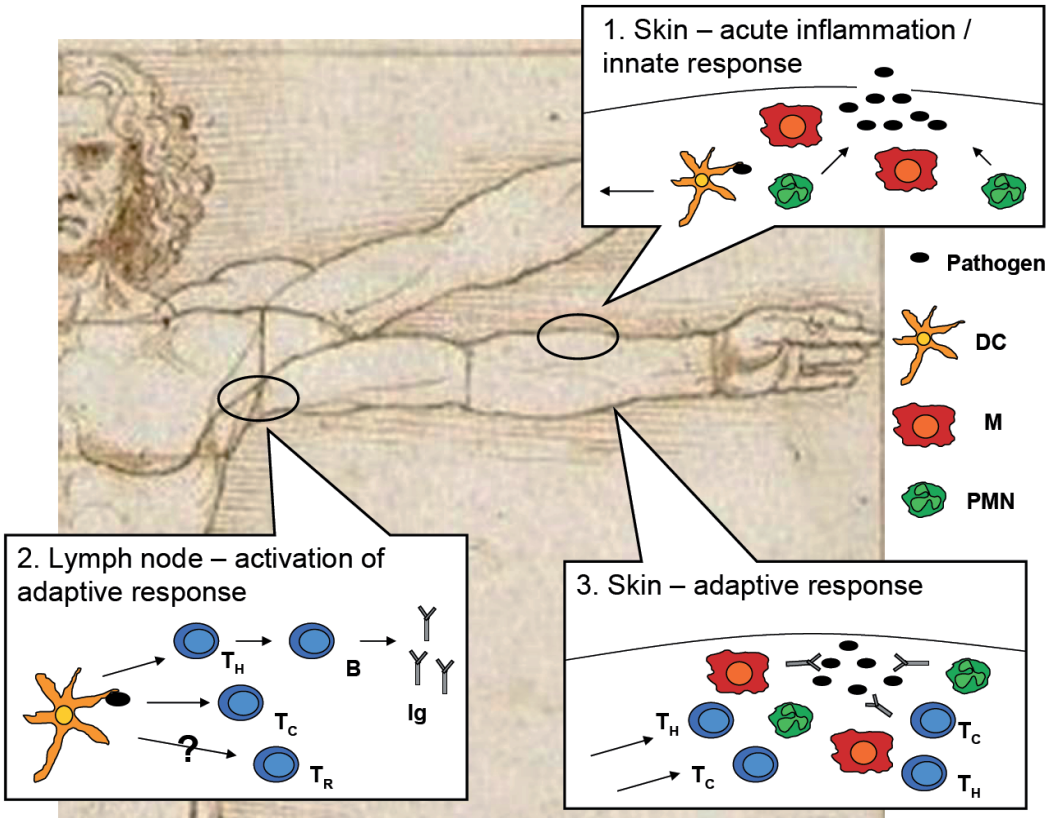

Figure 1. Simplistic view of the immune response to a skin infection. 1. A pathogen will need to break the skin barrier before establishing a focus for infection. Tissue-resident macrophages (M) recognize the intrusion and start the inflammatory response, including recruitment of polymorpho-nuclear neutrophil granulocytes (PMN). If this initial response is unable to clear the pathogen tissue-resident dendritic cells (DC) will carry antigens from the invading pathogen to the nearest lymph node. 2. In the lymph node, dendritic cells will activate helper T-cells (T_H), cytotoxic T-cells (T_C) and possibly regulatory T-cells (T_R) to activate and modify the adaptive immune response. Some T_H-cells will activate and mature B-cells in the lymph node, leading to the massive production of immunoglobulins (Ig). 3. T_H- and T_C-cells will migrate to the inflammatory focus to help combat the pathogen, as will the circulation of specific immunoglobulins. The nature of this immune response will be modified according to the features of the invading pathogen.

out the immune response by example of the response to an invasion of a pathogen to the skin (Figure 1).

The introduction of the pathogen to the human body relies firstly on the penetration of one of our main barriers to infection, the skin. This penetration is often, if not always, facilitated by the disruption of the skin barrier through a cut or other injury. When pathogens like e.g. bacteria have crossed this epithelial barrier they will localize and grow in the tissue close to the penetrating site causing *inflammation*. Local anti-infectious agents like peptides, proteins, plasma proteins, macrophages and granulocytes will then try to destroy and contain bacterial growth through the so-called *innate mechanisms of immune defense*. The innate immune response is so tightly inter-woven with the process of inflammation that some authors even claim it is a misnomer; the innate immune response cannot be separated from acute inflammation and thus merits no separate name. Semantics aside, these innate immune mechanisms are conserved through evolution with remarkable penetrance in a wide range of organisms, and represent congenital and stable responses to foreign substances. The so called natural killer (NK) cells are also considered a part of this innate immune response. The amplification and coordination of this, as well as most other cellular responses in the immune system, relies on the secretion and recognition of cell signaling substances called cytokines between the participating cells. In this first encounter macrophages will secrete TNF-α, IL-1 and IL-6 among other mediators of inflammation (such as prostaglandins and leukotrienes). While resident macrophages will encounter the pathogen first, the immediate response is more dependent on the migration of granulocytes to the site of inflammation by way of chemotaxis; a process of cellular movement towards a gradient of cytokines with a particular propensity for migration, *the chemokines* (e.g. IL-8 and MCP-1). This local response will furthermore facilitate the uptake of antigen, constituents from the invading pathogen able to elicit an immune response, by dendritic cells which will migrate to the closest lymph-node to present these antigens to T lymphocytes. This presentation marks the activation of *the adaptive immune system*.

In contrast to the well preserved and stable genetic codes at the base of innate immunity, adaptive immunity is based on the ability of lymphocytes to recombine their genetic material to adapt to challenge by foreign subjects. The T lymphocytes will, depending on the way in which these antigens are presented, respond by clonal expansion and differentiation of T- and B cells, leading to tender and swollen lymph

nodes. T cells are conventionally divided into the cytotoxic $CD8^+$ cells and the $CD4^+$ helper T cells, T_H1 and T_H2 cells. While the $CD8^+$ T cells are capable of eliminating cells infected with intracellular pathogens like virus, the $CD4^+$ cells have a more complex role orchestrating the immune response by way of regulating and activating other immune cells. Most T_H1 effector cells migrate to the focus of infection to help activate yet more macrophages through secretion of IFN-γ, IL-2 and TNF-β. In contrast, the T_H2 cells migrate within the lymph node to activate B cells by cell-to-cell contact and secretion of IL-4, IL-5 and IL-6. When B cells are activated, they will mature into plasma cells, producing vast amounts of antibodies capable of targeting pathogen antigens for destruction by macrophages, complement proteins and granulocytes. The combined and coordinated response of all constituents of the immune system represents a powerful and usually devastating response to any pathogen trying to invade the organism. This impressive *tour de force*, however, comes with a price; collateral damage of cells and tissues may emerge if this process of innate and adaptive immune responses is without strict *regulation*. This regulation is facilitated by several factors, including the activation of the so-called regulatory T-cells (T_{Reg}).

Lymph nodes are stationed at strategically important sites surveilling incoming lymph from e.g. the limbs. If an infection is not contained in the local tissue or controlled by the local lymph nodes, pathogens may spread through the blood, creating other sites of infection or directly infecting the blood causing sepsis. The lymphoid organ responsible for the surveillance of circulating blood is the spleen, containing white pulp that has many overlapping functions with regular lymph nodes. Spleen and lymph nodes are collectively called secondary lymphoid organs in contrast to the primary lymphoid organs of bone marrow and thymus with an active role in the development of lymphoid cells.

Infections manifesting themselves as disease and combated through the adaptive immune system likely represent a small fraction of the breaches of integrity due to infectious organisms but contained through innate mechanisms of immune defense. If the innate response fails to contain the pathogen, the level of pathogens will rise to a threshold where the innate response activates the adaptive response and both parts of the immune system act in concert to eliminate the pathogen (Figure 2). Notably, while symptoms of disease in infected tissues correlate with the level of

pathogens, symptoms are often more dependent on the immune response to the pathogen than on the pathogen itself.

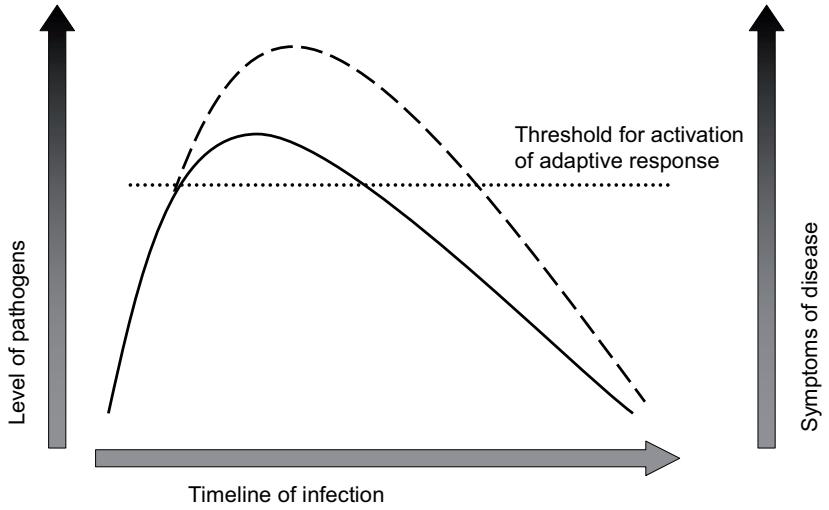

Figure 2. Theoretical model of contribution of the innate and adaptive responses during an infection with a pathogen. The innate response (whole line) is the initial response to the pathogen and will in most cases contain and destroy the pathogen. If the pathogens succeed in reaching a considerable number, they will pass a certain threshold where the innate response activates the adaptive response (dotted line) and both parts of the immune system act in concert to eliminate the pathogen. Most pathogens will be eliminated asymptomatically by the immune system and not cause overt disease.

1.1.1 Inflammation

Inflammation is primarily a protective response to cellular injury, from various trauma to infection, and is a basic and underlying process to the immune response, with the aim of restoring homeostasis to the affected tissues. Any inflammatory response can be seen to follow a pathway consisting of inducers, sensors, mediators and effectors. The different inducers will be recognized by more or less specific sensors leading to the release of mediators activating effectors, e.g. pathogens will be recognized by receptors leading to the release of cytokines activating effector cells. Inflammation has traditionally been defined as either acute or chronic and the definition of these merits some comments. The acute inflammation lasts from minutes to days, involve local tissue and innate mechanisms of immunity, including granulocytes. Chronic inflammation, on the other hand, is a process involving adaptive mechanisms of immunity, typically including lymphocytes and macrophages, and lasting days to years. Histologically, acute inflammation is characterized by the presence of an inflammatory exudate containing edema and a predominantly neutrophil infiltrate (2). Chronic inflammation, on the other hand, is characterized by infiltration of mononuclear cells (macrophages, lymphocytes), tissue destruction and repair (angiogenesis, fibrosis)(2). Biochemically, acute inflammation is characterized by the acute phase response involving high levels of local and systemic mediators like cytokines while these typically are more modestly expressed in chronic inflammation.

The process of acute inflammation, with some important exceptions like allergic anaphylactic reactions, is generally considered a beneficial response for restoring tissue integrity. Chronic inflammation has a more ambiguous role; while it is an appropriate response leading to clearing of many important pathogens and resolution of the inflammatory state it may also be inappropriate leading to tissue damage with no apparent benefit to the host. This inappropriate activation is characterized by either a sustained response to an already cleared or contained pathogen, an erroneous response towards an innocuous antigen or a response towards self-antigens as seen in autoimmunity. Furthermore, while localized chronic inflammation has a clear role in diseases like tuberculosis, rheumatoid arthritis and silicosis mounting evidence also suggests a role for systemic chronic inflammation with largely negative effects in diseases like obesity and diabetes. These diseases are characterized by inflammatory traits albeit not of the nature usually seen in classic

inflammatory diseases. Thus, there is no focus of chronic inflammation and there is a low-grade response with low raise of systemic levels of inflammatory mediators. Recently, a state of para-inflammation was suggested as an intermediary step between tissues in a homeostatic equilibrium and the classic chronic inflammation, encompassing diseases where tissue malfunction, and not overt injury, would lead to (sub-)inflammatory responses (5).

A central cell in the inflammatory response is the macrophage, acting as sensor, mediator and effector. Tissue-resident macrophages form a considerable part of most tissues and are known to have a central role surveilling peripheral tissues for possible intrusions like infection or trauma. However, they are also known to have a role controlling tissue homeostasis through phagocytosis of apoptotic cells and release of homeostatic mediators. The involvement of macrophages in inflammation can thus include both activation of the inflammatory response and adaption of tissue homeostasis to a new equilibrium. While macrophages in the basal state share many characteristics, activation of macrophages can lead to the development of functionally different classes of macrophages, recently described as classically activated, wound healing and regulatory macrophages(6). Common for all of these macrophages is the ability to respond to low intensity stimuli in the innate part of inflammation. The activation of macrophages is thus a sensitive marker of local tissue stress as seen in low-grade chronic inflammatory states(7).

1.1.2 Innate immunity

The development of multi-cellular organisms required potent mechanisms of defense against destruction, and mechanisms of host defense can be seen in lower organisms such as plants and non-vertebrates. Many of these phylogenetically old mechanisms are preserved in higher animals and humans, but some have possibly been rendered redundant by the evolution of an increasingly effective immune system. This is, as I will discuss later, of particular interest in the study of deficiencies of this immune system.

As previously mentioned, the innate mechanisms of immune defense are closely interconnected with inflammation and, one could argue, inseparable from it. However, some processes have by convention been associated more with the stricter term of innate immunity than the more widely defined term of inflammation.

At the basis of innate immunity is the presumption that there are some common and stable traits on the surface of pathological microbes like bacteria, virus and fungi. These traits or structures are called pathogen-associated molecular patterns, (PAMP). These molecular structures change only minimally over time and are thus possible targets for recognition by the immune system through the so called pathogen recognizing receptors (PRR). Both PAMPs and PRRs comprise a wide range of structures that likely will continue to be redefined and extended as we gather more knowledge of our immune system. Broadly speaking, innate mechanisms of immune defense will include anything from physical barriers of the human body towards the exterior, namely skin and mucosa, to NK-cells. I will here go into detail regarding some important parts of the innate immune system.

Defensins and other anti-microbial peptides and proteins

A primitive part of the immune system is the anti-microbial peptides and proteins constituvely found on epithelial surfaces or released from leukocytes upon activation. The defensins, α-defensin and β-defensin, are among the best described and are cationic peptides that are thought to destroy the bacterial cell wall. Lactoferrin and transcobalamin can bind iron and vitamin B12, respectively, vital nutrients to bacteria and thus inhibit bacterial growth. Another protein with recently described microbicid function is the urokinase plasminogen activator (uPA) that has been shown to act as a systemic endogenous antibiotic (8).

The complement system and mannose-binding lectin

The complement system, consisting of more than 30 proteins, represents an important component of the innate immune system. The hierarchical cascade of complement activation from any of three different starting points all lead to a final common pathway capable of neutralizing toxins, microbes and cells either directly through the membrane-attack complex or by opsonisation of pathogens (Figure 3). Split products of this process, the so-called anaphylatoxins C3a, C4a and C5a, all act as mediators of inflammation contributing to the establishment of the inflammatory exudate, including recruitment of inflammatory cells. While the complement system plays a central role in the host defence against microbes, enhanced complement activation may also induce tissue damage and inflammation, through activation of both the terminal complex and the production of anaphylatoxins. Accordingly, a dysregulated

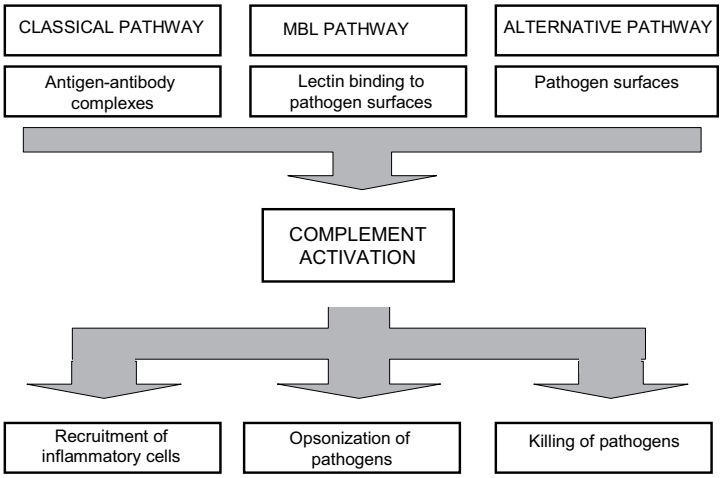

Figure 3. Overview of the complement system. The complement system can be activated through three different pathways all leading to recruitment of inflammatory cells, opsonization and killing of pathogens. Adapted from (4).

complement system has been associated both with increased susceptibility to infections and autoimmune diseases (9;10).

Mannose-binding lectin (MBL) is the key component of the lectin pathway of the complement system, binding to sugar residues on the surface of pathogens and marking them for destruction. Serum levels of MBL are closely correlated to polymorphisms in promotor regions as well as mutations in the MBL gene (11-13).The extent of these malfunctions of the MBL gene is varying in different populations with high penetrance in tropic populations and lower penetrance in temperate and arctic populations. This has led to speculation that MBL deficiency may be of some value in tropic regions while a fully functioning MBL gene may be of benefit in more temperate regions. While any value of MBL deficiency is unclear, the benefit of MBL may be related to protection against particularly airway pathogens, which possibly is of importance in temperate and arctic regions. Some studies have found that children with recurrent sinopulmonary infections have low levels of MBL (14;15), but low MBL levels do not seem to increase mortality or the occurrence of

infectious disease in an adult population (16). On the other hand, altered MBL levels have been associated with persistent inflammation and tissue destruction, potentially contributing to the pathogenesis of some inflammatory disorders (17;18).

Toll- and other -like receptors

The fruit-fly *Drosophilia melanogaster* has a receptor called Toll central in its defense against pathogens. In humans, receptors similar to Toll, the Toll-like receptors (TLR), are equally found to be immunologically active. As of today, 13 different receptors (TLR1-TLR13) have been identified, classified in 5 groups based on their structure. The receptors are expressed on a wide variety of cells from dendritic cells to B cells and respond to pathogens like Gram-negative bacteria (TLR4), Gram-positive bacteria (TLR2) and viruses (TLR3). TLR9 deserves special attention, it is together with TLR3, TLR7 and TLR8 located intracellularly. Furthermore, TLR9 is located in B cells and stimulated by CpG DNA, a DNA variant found in bacteria. The TLRs play a central part in the communication between the innate and the adaptive immune system, notably in the peripheral activation of dendritic cells and macrophages. The differential activation of TLRs as well as probable co-receptors lead to secretion of both inflammatory and anti-inflammatory cytokines and can modulate the T-cell response in a T_H1 or T_H2 like manner (19). It is also suggested that TLR activation can lead to the production of anti-microbial peptides.

The discovery of intracellular TLRs has been followed by recognition of other innate intracellular receptors like the nod-like receptors (NLR). NLRs recognize bacterial constituents within the cell as well as other danger signals leading to activation of the cell.

1.1.3 Adaptive immunity

The presentation of antigens to lymphocytes marks the involvement of the adaptive immune system in the immune response. The ability of lymphocytes to recombine its genetic material to match an almost indefinite number of antigens is the primary force of this response. This adaption of the immune response leads to a specific attack on the antigen, and equally important, this adaption to the acute incident can be preserved throughout life with the development of immunological memory. Cellular immunity through the involvement of $CD8^+$ T cells will not be commented upon here

as it is the humoral immunity based on the activation of $CD4^+$ T cells and B cells that is the theme of this thesis.

Antigen-presenting cells (APC) like dendritic cells present antigens to $CD4^+$ T cells in the T-cell zones of the lymph node. The activation of these T cells requires a T-cell receptor with avidity for the presented antigen as well as co-stimuli in the form of binding to the CD28 and CD4/8 molecules on the T cells by the B7 (CD80 and CD86) and MHC II molecule on the APC, respectively. CD40L is also presented on the T-cell binding to the APC expressed CD40. Another important receptor for T-cell activation is CTLA-4 (CD152), a receptor resembling the B7 molecules but with higher avidity. Furthermore, the inducible co-stimulator (ICOS) is expressed on activated T cells and bind to its ligand (LICOS) present on dendritic cells, but importantly also on B cells which will be of interest to this discussion.

The nature of T-cell activation will determine whether the $CD4^+$ cells develop into T_H1 or T_H2 effector cells, and consequently deciding whether the response to the offending antigen will be cellular (T_H1) or humoral (T_H2), profiled to combat either intracellular or extracellular pathogens, respectively. The mechanisms of this process are not fully known but some cytokines have an established role; the presence of IFN-γ and IL-12 will lead to a T_H1 response, while IL-4 leads to a T_H2 response. Furthermore, the phenotype of dendritic cells is known to be of importance as myeloid DC preferentially induce T_H1 cells while the so-called plasma-cytoid DC have a propensity for T_H2 cells. While most activated T_H1 cells will migrate to peripheral tissues some will, together with the T_H2 cells, remain in the lymph node to activate B cells. The activation of B cells requires recognition of the same antigen, but not exactly the same epitope, as that on the T-cell as well as contact between these cells. The binding of CD40L on the T-cell to CD40 on the B-cell is of importance, as well as T-cell production of IL-4, IL-5 and IL-6. Other signals include BLyS which binds TACI expressed by T cells. The initial contact and activation of B cells will be in the primary focus in the T-cell zone of the lymph node. Activation of B cells here will lead to the production of IgM but further activation will lead to migration into the B-cell zone of the lymph node called a primary lymphoid follicle. The presence and proliferation of activated B cells in this follicle will lead to the establishment of a germinal centre(7). In this germinal center, activated B cells with the continuing help of T cells, will refine the humoral response through isotype switching and somatic hypermutation.

The end product of the humoral defense system, the antibodies IgA, IgM, IgG and IgE is of particular interest in this thesis as it is the loss of one of them, namely IgG, that defines the disease at consideration and as the therapy available to these patients is the replenishment of the same antibody through intravenous infusion. Different types of antibodies have distinct localizations and functions. Antibodies have at least four major modes of function, all through their binding to antigens;

- Neutralization through blocking of adhesive or toxic epitopes of the antigen (IgG, IgA)
- Opsonization through binding to the surface of pathogens and subsequent binding of the antibodies to FC receptors on phagocytes (IgG)
- Activation of complement through binding of antibodies to the C1 complex. This may lead to either activation of the complement cascade or to binding of the C1 complex to the complement receptor present on phagocytes and erythrocytes (IgM, IgG).
- Sensitization of mast cells. Mast cells bind, in contrast to all other cells, unbound IgE antibodies through their FC receptors. The binding of the surface anchored antibody will then lead to activation of the mast-cell (IgE).

The opsonizing function of IgG is particularly important in defense against polysaccharide capsuled bacteria as this capsule serves as a protection against non-antibody mediated phagocytosis.

1.1.4 Chemokines and chemotaxis

Communication between cells not in contact depends on the cytokine network, a large group of soluble proteins active in a paracrine manner. Chemokines, *chemotactic cytokines*, are a group of cytokines contributing to the migration of leukocytes between different cellular compartments like inflamed tissue, secondary lymphoid organs and the circulation (Table I). They act together with integrins capturing leukocytes in the circulation and leading them through the extra-cellular matrix. Functionally, chemokines have been divided between those associated with inflamed tissue, the inflammatory chemokines, and those seen in secondary lymphoid organs, the so called homeostatic chemokines. Not surprisingly, this dichotomy of nomenclature is challenged by ever increasing evidence of overlapping functions between chemokines. Structurally, chemokines have now been named and defined

based on the structure of a cysteine (C) motif in the N-terminal end of the amino acid sequence, and three major classes have been described; the CC, CXC and CXXXC (or CX3C) classes, where the X represents the presence of a non-cysteine amino acid (20). In all classes chemokines are named numerically, even if some names preceding this classification system are still in use. The chemokines act through activation of the chemokine receptors, a family of G-coupled receptors, named correspondingly to the chemokines as CCR, CXCR and CX3CR, respectively. While some chemokine receptors like CCR2 are present on a wide variety of cells, others are presented more restrictively on subsets of leukocytes and have emerged as co-markers of certain subsets of leukocytes. A key feature of the chemokine network, however, is its redundancy with overlapping functions between chemokines and receptors. Notably, most receptors can be stimulated by more than one chemokine.

Family	Systematic name	Original name	Target cell	Receptor
CCL	5	RANTES	Monocyte, macrophage, T-cell, DC, NK cell	CCR1, CCR3, CCR5
	2	MCP-1	T-cell, monocyte, basophile	CCR2
	19	MIP-3β	Naïve T-cell, mature DC, B-cell	CCR7
	21	6Ckine	Naïve T-cell, B-cell	CCR7
CXCL	1	GRO-α	Neutrophil	CXCR2, CXCR1
	8	IL-8	Neutrophil, basophile, T-cells	CXCR1, CXCR2
	13	BLC/BCA-1	B-cell, activated CD4+ T-cell	CXCR5
CX3CL	1	Fractalkine	T-cells, monocytes	CX3CR1

Table I. Selected chemokines. Chemotactic cytokines responsible for cytokine migration in both homeostasis and inflammation. Chemokines are now named and defined by the structure of a cysteine (C) motif in three classes; CCL, CXCL and CX3CL, as are their receptors; CCR, CXCR and CX3CR, respectively. The use of their original name is common (and sometimes confusing). DC = dendritic cell, NK cell = natural killer cell.

In addition to leukocytes, the most important cellular sources of chemokines are endothelial cells and various stromal cells, and there are also reports on chemokine receptors being expressed on cells other than leukocytes, like smooth muscle cells.

Apart from propagation of migration, chemokines have stimulatory and regulatory effects on their target cells. This has important implications; first it establishes chemokines as central mediators of the immune response, secondly systemic levels of chemokines will not only be reflecting a local immune response in lymph nodes or peripheral tissue, they will directly affect the state of cells encountered in the circulatory compartment. Moreover, chemokines may affect non-leukocytes cells like smooth muscle cells and endothelium.

In the same way that chemokines have potential for more than inducing chemotaxis, proteins from other families can have chemoattractive properties. The urokinase plasminogen activator (uPA) is a member of the fibrinolytic system, but has together with its receptor uPAR been shown to promote chemotaxis of phagocytes and T cells. While regular chemokines stimulate chemotaxis through remodeling of the cytoskeleton, uPA exhibits an additional feature through the local proteolysis initiated by the binding of uPA to uPAR.

1.1.5 Regulation of the immune response

The appropriate responses of the immune system rest as much on tolerance of harmless antigens as on immunity against harmful antigens. This tolerance of harmless antigens is attributed primarily to the adaptive immune system and in particular the T cells. Central tolerance is mediated through mechanisms in primary lymphoid tissue and includes deletion of auto-reactive T cells. Peripheral tolerance includes anergy, suppression and immunological ignorance, and operates in secondary lymphoid tissue and peripheral tissues. These mechanisms of tolerance can be attributed to different steps in the immunological response and to different T-cell types. While e.g. anergy is dependent on the incomplete activation of effector T cells, suppression reflects activation of specific cells with a potential for what has lately been named regulation, rather than suppression.

Several cell types have regulatory properties, although some are poorly described;

- Tr1; induced by antigen-stimulation, secretion of the modulatory cytokines IL-10 and TGF-β.
- T_H3; induced by oral antigens, secretion of TGF-β.
- γδ T cells; naturally occurring in mucosa, loss of these cells associated with autoimmunity.
- NKT cells; surface markers for both for both T- and NK-cells.
- $CD8^+$ T_{Reg}; uncertain identification.
- $CD4^+CD25^+$ T_{Reg}; naturally occurring regulatory T cells.

The identification and study of $CD4^+CD25^+$ regulatory T-cells has been a breakthrough in our knowledge of peripheral tolerance. The removal of thymus from mice 3 days old was shown to lead to autoimmune disease while the simultaneous transplantation of $CD4^+CD25^+$ cells alleviated this. Further examination of these cells found a subgroup expressing high levels of the IL2 receptor CD25 and the transcription factor FoxP3 with potent regulatory properties (21;22). These $CD4^+CD25^{High}FoxP3^+$ cells have been called naturally occurring regulatory T cells(T_{Reg}), as they, in contrast to e.g. Tr1 and T_H3 cells, have suppressive properties independent of antigen stimulation. However, it seems clear that there also are inducible T_{Reg} expressing the FoxP3 factor, and as FoxP3 has also been found in CD8+ cells with a regulatory capacity, some claim that FoxP3 is a lineage marker for cells with regulatory functions. Interestingly, it has also been suggested that regulatory function is determined by higher-order processes that ensure the expression of FoxP3 (23). In any case, the importance of the identification of FoxP3 has been underscored by the fact that mutation of this transcription factor is associated with immune dysregulation and autoimmunity in both mice and man (24). Furthermore, while the co-expression of CD4 and $CD25^{High}$ previously has been used as a phenotype marker for T_{Reg}, the identification of FoxP3 now allows for a more strict and valid classification of T_{Reg} even in clinical studies (25).

The biological role of T_{Reg} is to suppress activity of primarily effector cells although it seems T_{Reg} are also capable of dampen responses in antigen-presenting cells like DC and B cells (26). The T_{Reg} use a variety of mechanisms to achieve this suppression, including killing of cytotoxic cells through cell-to-cell contact as well as secretion of anti-inflammatory cytokines like TGF-β and IL-10 (27-29). The proper activation of T_{Reg} will require signals from peripheral tissues, coordinating the

activation of T_{Reg} with the need for a down-regulation of the immune response, and are probably delivered through antigen-presenting cells like dendritic cells.

1.1.6 Interaction and cooperation of innate and adaptive immune responses

The traditional dichotomy of immune responses in either innate or adaptive is challenged by the increasing evidence of interaction that exists between the constituents of the immune system. It has long been known that innate responses are crucial for initiating adaptive immunity, and that the effector cells of the adaptive immune response uses innate mechanisms to amplify their response to the offending antigen. However, recent evidence suggests that innate immunity continues to modify the adaptive response also after antigens are presented and the adaptive cells are activated. Antiviral T cells will maximise their response if they are provided with innate stimuli also during the immune response, and the same signals will promote survival of effector cells (30). On the other hand, antigen-presenting cells are capable of down-regulating T-cell responses and proliferation, suggesting innate immune mechanisms are involved in regulating the contraction phase of the T-cell response (31).

1.2 PRIMARY IMMUNODEFICIENCIES

The malfunction of one or more constituents of the innate or adaptive immune system may, despite the large degree of overlapping functions and redundancy of individual factors, lead to a clinical immunodeficiency. Importantly, immunodeficiencies can manifest themselves not only through increased susceptibility to infectious diseases but also through increased frequency of malignant disease and, more intriguingly, autoimmunity. Immunodeficiencies have traditionally been classified as either primary, due to an unknown cause or congenital genetic defect, or secondary, due to exposition to an immunosuppressive agent in the form infections, drugs, radiation or other. The advent of the HIV epidemic in the 1980's suddenly made immunodeficiency a major contributor to human mortality and morbidity, and HIV and other secondary forms of immunodeficiencies, including iatrogenic variants, continue to rise in prevalence. However, there has also been a significant development in the smaller field of primary immunodeficiencies in later years, identifying yet more causes of immunodeficiencies and exploring them and previously described immunodeficiencies on the molecular level. The study of these syndromes may cast light also over more frequently occurring diseases.

Primary immunodeficiencies can be caused by defects in both the innate and adaptive immune system, and so far over 150 different deficiencies have been described (32)(Table II). They span over a vast clinical spectrum with many of the most severe forms related to T-cell defects, while other forms seem to have little or no certain clinical consequence. Some primary immunodeficiencies exhibit a classic autosomal recessive or X-linked genetic inheritance, but in many forms the genetic cause is complex and probably also influenced by as of yet unknown environmental factors. Humoral immunodeficiencies, either through isolated B-cell defects or as a result of T-cell defects, constitute the majority of cases.

Localization of deficiency	Subgroup	Name	Inheritance	Clinical severity
Innate	Complement	C2-deficiency	Autosomal recessive	++
		MBL-deficiency	Complex, autosomal recessive	-(?)
	Phagocytes	Chronic granulomatous disease	Complex, X-linked	++
Adaptive	T-cells	Severe combined immunodeficiency (SCID)	Different genetic causes, most X-linked	+++
		MHC I deficiency	Autosomal recessive	+
		MHC II deficiency	Autosomal recessive	+++
	B-cells	IgA deficiency	Autosomal recessive	+
		Hyper IgM syndrome	Complex, most X-linked	++
		Brutons/X-linked agammaglobulinemia (XLA)	X-linked	++
		Common variable immunodeficiency	Complex	++

Table II. Selection of primary immunodeficiencies. Immunodeficiencies can affect almost any constituent of the immune system, some are asymptomatic while others are severe and fatal.

1.2.1 Common variable immunodeficiency

Diagnosis

Hypogammaglobulinemia can occur secondary to other immunological or non-immunological diseases but the hallmark of CVID is the absence of IgG-antibodies not related to any other known disease. The many causes of hypogammaglobulinemia makes the definition and diagnosis of CVID a matter of exclusion, and this is furthermore complicated by the heterogeneous nature of both clinical symptoms and etiology in CVID. The WHO expert group on primary immunodeficiencies, the IUIS (International Union of Immunological Societies) scientific committee, has defined the diagnosis of CVID as being defective antibody formation with decreased serum levels (>2 SD) of IgG, IgA and/or IgM, together with exclusion of other forms of hypogammaglobulinemia, most notably the Hyper IgM syndrome and Brutons agammaglobulinemia (X-linked agammaglobulinemia, XLA)(33). Others use a stricter definition, further requiring age at onset of symptoms to be over 2 years and poor antibody responses (i.e. absence of isohemagglutinins or poor response to vaccines)(34).

Epidemiology and etiology

CVID is the most prevalent primary immunodeficiency with an estimated prevalence of 1:50 000 to 1:200 000 (32) and with an equal sex distribution. The disease usually manifests itself in the third decade although there is a wide range of age at debut (35;36), with diagnosis typically delayed by several years. CVID is associated with significantly increased mortality, even if survival data seem to be improving (36). No clear pattern of inheritance has been established in CVID and most cases seem to be of a sporadic nature, even if the disease has been seen to cluster in some families. In recent years, four genetic defects affecting co-stimulatory molecules in B and T cells (ICOS, CD19, BAFF-R, TACI) leading to CVID have been identified on a molecular level. These genetic defects only accounts for a fraction of all cases and each will be discussed in more detail later. However, it is noteworthy that all defects concern co-stimulatory molecules illustrating the role of co-stimulation in the immune response. Other possible etiological agents include infections and certain medications although there is no proven association between these factors and CVID.

Clinical features

CVID patients can present a variety of symptoms from different organ systems (Figure 4). Recurrent sino-pulmonary infections are the most common clinical manifestation of CVID, affecting nearly all patients (35). These infections may be complicated by the development of chronic sinusitis, chronic otitis and bronchiectasis. Bronchiectasis represent the most common pulmonary complication, affecting >25% of patients, and it is associated with increased mortality (36). Common pathogens are encapsulated bacteria like *H.influenzae* and *S. pneumoniae* highlighting the importance of opsonizing by IgG in the defense against these bacteria. However, patients are also prone to infections with atypical bacteria like *Mycoplasma spp* (32). Gastrointestinal infections by *Salmonella, Campylobacter* and G*iardia* are over-represented in the CVID population as well as enteroviral infections. The CVID population shares many of these pathogens with XLA patients pointing to the protective role for IgG in immune defense against them. In contrast, while CVID patients are prone to severe *Herpes Zoster* infection this is rarely seen in XLA, possibly reflecting the more profound immune deficiency seen in some CVID

patients. The aggressive course of *Hepatitis C* infection in CVID patients as compared to XLA further underscores this difference (37).

Malignant diseases, both solid and non-solid tumors, affect a disproportionate part of the CVID population, most strikingly Non-Hodgkin lymphomas affecting approximately 3-7 % of patients but there is also an increased risk of gastric carcinomas (35;36).

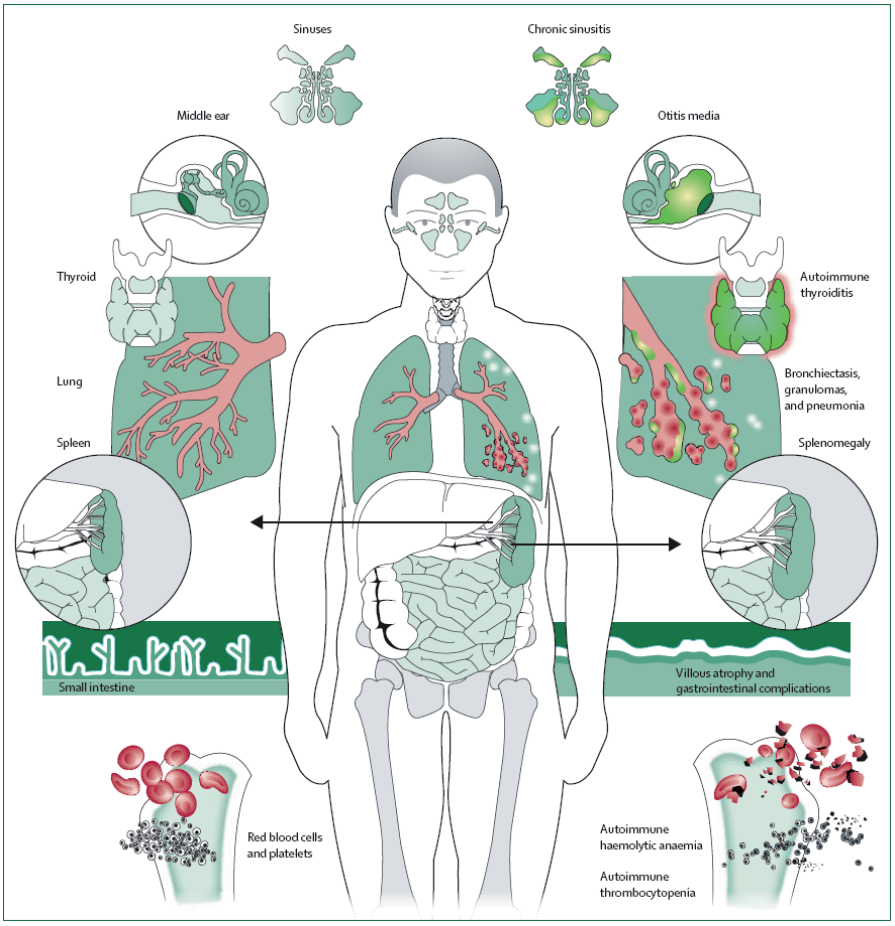

Figure 4. Organ systems involved in the pathogenesis of CVID. Left; healthy organs. Right; organ-system involvement. By permission from (32).

CVID is characterized by a high frequency of autoimmune features, in particular idiopathic thrombocytopenia and autoimmune haemolytic anemia (6-7% and 4-5% of patients, respectively), which in some cases mark the debut of the disease (35;36). Other autoimmune features include pernicious anemia, atrophic gastritis, autoimmune thyroiditis and rheumatoid arthritis. Furthermore, villous atrophy and anti IgA antibodies is seen in a number of patients, and non-infectious chronic diarrhea is a common problem.

Benign lymphoid hyperplasia is seen in many patients with splenomegaly being the far most common manifestation (30-40% of patients)(36;38;39). However, nodular intestinal lymphoid hyperplasia is also present. The etiology of lymphoid hyperplasia in CVID is unknown, and possible causes would include chronic infection with low-virulent pathogens. On the other hand, CVID patients have an increased risk of developing lymphomas and lymphoid hyperplasia could thus also be seen as a feature related to intrinsic lymphocyte defects. Furthermore, non-caseating granulomas are also seen in lungs, spleen and the gastrointestinal tract independently of lymphoid hyperplasia (32;40). These granulomas represent a probably under-reported complication of CVID as it rarely gives symptoms and only can be diagnosed through biopsy of the affected organ. They are suggestive of a classic chronic infectious focus but no pathogen has so far been isolated, raising the possibility of an autoimmune focus. In any case, the granulomas could be seen as the result of failure of feedback mechanisms normally constraining lymphocyte proliferation.

The heterogeneous clinical picture of CVID reflects not only the various defects behind the hypogammaglobulinemia, but also the profound perturbation of the immune system some of these separate defects may lead to.

Immunological features – general consideration

The fact that many CVID patients have B cells with appropriate response to stimulation *in vitro* demonstrate that while the hypogammaglobulinemia *per se* affects the adaptive immune system, other defects in the closely interacting chain of events leading up to an appropriate antibody response may cause CVID (41). The search for these defects are complicated, not least due to the fact that any defect causing hypogammaglobulinemia as well as the hypogammaglobulinemia itself, can affect other parts of the immune system, making the distinction between primary and

secondary changes to the immune system in CVID difficult. However, in general, CVID is characterized by features of systemic inflammation, including raised circulatory levels of inflammatory cytokines and markers of oxidative stress (42-45).

Features of innate immunology

The essential function of the innate immune response is to remove intruding agents and if this fails, to activate the adaptive immune response through presentation of antigens to T cells. In CVID, innate immunity may be of particular importance to clear as many pathogens as possible through responses independent of the adaptive immune system. Furthermore, the processing and presentation of antigens by the innate response affects both magnitude and specificity of the adaptive response. Dendritic cells are the most potent of antigen-presenting cells and both quantitative and qualitative abnormalities have been seen in CVID. CVID patients are characterized by low levels of dendritic cells, both within the myeloid and plasmacytoid subset (46-48), stimulating T_H1 and T_H2 responses, respectively. As mentioned earlier, activation of T_H1 effector cells lead to a cellular immune response while a T_H2 response would cause a predominantly humoral response. Thus, the antibody deficiency of CVID could stem from an imbalance in the regulation of these two responses. There are data supporting an upregulation of the T_H1 response in CVID through increased serum levels of IL-12, as well as enhancement of the IL-12/IFN-γ cytokine loop in monocytes and T cells (47;49;50). Dendritic cells from CVID patients however, produce low levels of IL-12 as compared to controls (51;52). Dendritic cells from CVID patients furthermore show immature characteristics with deficient presentation of receptors relevant for T-cell stimulation (51;53). Interestingly, impaired differentiation of dendritic cells in CVID has been associated with low levels of circulating antibodies (54).

While there is no quantitative change of monocytes in CVID, patients have activated monocytes as reflected by increased serum levels of neopterin as well as enhanced generation of reactive oxygen species and spontaneous TNF-α release (43;45;55). Interestingly, while these monocytes are activated *in vivo* their response to stimulation *in vitro* seem hampered, suggesting this inappropriate activation is combined with a failure of adequate response to stimuli (45).

There are some studies of single complement components in CVID, but no systematic study of complement deficiency and CVD has been reported (56;57). This

paucity of studies also applies to other constituents of the innate immune system which represents novel and interesting approaches to a disease defined by failure of the adaptive immune response.

Features of adaptive immunology – T cells

Various T-cell abnormalities in CVID have been reported for many years including low expression of the co-stimulatory molecule CD40 ligand which is essential for B-cell activation. However, the discovery of ICOS-deficiency as a cause for CVID was a breakthrough (58). It established that T-cell defects could be a cause for CVID and it was the first genetic defect shown to cause CVID. ICOS is expressed mainly on activated T cells and is activated by ICOS-ligand expressed on B cells and antigen-presenting cells releases. The activation of ICOS leads to the secretion of several important cytokines from the T-cell, but it seems to be pivotal for the secretion of IL-10 and expression of CXCR5 (59). While the expression of CXCR5 is essential for the co-localization of B and T cells in the lymph node, IL-10 is needed for the terminal differentiation of B cells. Thus, ICOS deficiency attenuates the stimulatory interaction between otherwise normal B and T cells. ICOS-deficiency is an autosomal recessive genetic defect and so far nine patients have been described (32). The effects of the ICOS deficiency parallels that of the low expression of the co-stimulatory molecule CD40 ligand previously described in T cells from some CVID patients (60).

Phenotypically, the T-cell population in CVID has a decreased CD4/CD8 ratio, as well decreased proportions of $CCR7^+$ and $CD45RA^+$ T cells characterizing a shift from a naïve towards an inflammatory T-cell phenotype (61-66). Other features include attenuated proliferative response to mitogens *in vitro*, as well as increased proportions of apoptotic markers (35;67). T cells from CVID patients further fail to show antigenic specificity after vaccination (68). Thymic output of naïve lymphocytes is reduced while peripheral proliferation of both $CD4^+$ and $CD8^+$ T cells are increased (67;69).The cytokine profile in stimulated T cells from CVID patients reveal an increase in the levels of IFN-γ and a decrease in IL-10 (67;70), and growing insights into T-cell relevant cytokine networks makes this an interesting area of research. Furthermore, advances in the area of regulatory processes may be of potential relevance to T-cell mediated inflammation in CVID.

Features of adaptive immunology – B cells

As noted, many CVID patients seem to have normal B-cell function *in vitro,* and only a minority of patients has B-cell lymphopenia. However, both phenotypic and functional abnormalities have been observed in the B-cell population of CVID patients, some being causative of the disease.

While the B-cell lymphopenia in some patients suggests a defect in early B-cell differentiation, other CVID patients are characterized by low numbers of $CD27^{+}IgD^{-}$ switched memory B cells pointing to a defective terminal differentiation, possibly by failure of germinal centre development (39;71;72). TLR9 is an intracellular receptor that is a potent inducer of converting B cells to plasma cells. The ligation of CpG-DNA to TLR9 leads to proliferation and differentiation of B cells from healthy controls. In contrast, CVID patients show a quantitative and functional TLR9 defect not only in B cells but also in plasmacytoid dendritic cells (73).

The identification of the T-cell associated ICOS-deficiency as causative to CVID has been followed by the identification of several B-cell associated genetic defects leading to CVID. CD19 is used as a marker of B cells and is, in complex with other proteins and the B-cell receptor, essential for B-cell activation. There are now three CD19 mutations identified (74;75). Mutations have also been found in the APRIL-BAFF network which is essential for B-cell development and survival (59;76). B-cell activating factor receptor (BAFF-R)(TNFRSF13C) is expressed on naïve circulating B cells while Transmembrane activator and calcium-modulating cyklophilin ligand interactor (TACI)(TNFRSF13B) is expressed in germinal center B cells. The TACI mutation is the most prevalent, possibly affecting up to 10% of patients, but there is no clear association to a particular clinical phenotype (77;78). The involvement of the APRIL-BAFF network in CVID is further suggested through enhanced levels of the ligands to BAFF-R and TACI, namely APRIL and BAFF (79).

Classification

The heterogeneous nature of CVID has prompted several attempts to classify patients according to clinical or immunological criteria (36;39;41). The main challenge of classifying CVID is the relative weak association between immunological, clinical and genetic features, not least highlighted in the heterogeneous clinical picture of the CVID patients with the TACI-defect (78). As previously noted, some CVID patients are characterised by low numbers of isotype switched memory B-cells and in later

years several groups have proposed classifying patients according to this B-cell phenotype, most recently in the EUROCLASS trial (39). However, the clinical value of this classification is uncertain (36), and no consensus in classification of CVID has yet been reached.

In articles focusing on inflammatory characteristics in CVID we have previously described a subgroup of patients ($CVID_{Hyper}$) with chronic inflammation *in vivo* as reflected by splenomegaly and increased serum levels of the monocyte activation marker neopterin (42;43;55). This subgroup of patients, in contrast to the other patients classified as $CVID_{Norm}$, shows a consistent pattern of raised inflammatory markers and allows for a relatively robust classification of CVID patients.

2. PURPOSE OF THE STUDY

To explore the possible contribution of innate, inflammatory and regulatory immune mechanisms in the pathogenesis of CVID.

3. SUMMARY OF RESULTS

3.1 PAPER I; COMMON VARIABLE IMMUNODEFICIENCY AND THE COMPLEMENT SYSTEM; LOW MANNOSE BINDING LECTIN LEVELS ARE ASSOCIATED WITH BRONCHIECTASIS

The objective of this study was to evaluate mannose-binding lectin (MBL) and the complement system in relation to clinical and immunological parameters in patients with common variable immunodeficiency. Circulating levels of MBL, complement components, complement activation products and functional capacity of complement pathways were correlated to clinical features in CVID patients and compared with healthy controls. The main findings were;

- The patients had signs of increased complement activation significantly associated with signs of an inflammatory phenotype.
- There was no evidence of deficiencies of the classical and alternative complement pathways in the patient group.
- The prevalence of lectin pathway deficiency was the same in patients and controls, but patients with increased frequency of lower respiratory tract infections or bronchiectasis had lower capacity of the lectin pathway than patients without these features.
- The serum concentration of MBL was inversely correlated with the frequency of lower respiratory tract infections and bronchiectasis.

Conclusion; Patients with common variable immunodeficiency do not have increased frequency of complement deficiencies but signs of increased complement activation associated with an inflammatory phenotype. MBL and the lectin complement pathway may contribute to protection against lower respiratory tract infections in patients with common variable immunodeficiency.

3.2 PAPER II; ENHANCED LEVELS OF UROKINASE PLASMINOGEN ACTIVATOR AND ITS SOLUBLE RECEPTOR IN COMMON VARIABLE IMMUNODEFICIENCY

The urokinase plasminogen activator (uPA), its cell bound and soluble receptor (uPAR, suPAR) have complex biological functions involving innate immune defense mechanisms and regulation of inflammation. Based on this dual role, we hypothesized that the uPA system could be affected in CVID, and examined expression of components of the uPA system in subgroups of CVID. Circulating levels of uPA and suPAR were correlated to clinical features in CVID patients and compared with healthy controls. Monocytes from CVID patients were analysed for uPAR expression by means of flowcytometry. The main findings were:

- All CVID-patients had increased plasma levels of suPAR with particularly high levels in those with splenomegaly and thrombocytopenia.
- Plasma uPA levels were also raised in patients with splenomegaly and thrombocytopenia.
- suPAR and uPA levels correlated with circulating levels of the monocyte activation marker neopterin.
- Monocytes from CVID patients had increased expression of uPAR.

Conclusion; We show an inappropriate activation of the uPA system possibly contributing to the inflammatory phenotype seen in subgroups of CVID patients.

3.3 PAPER III; CHEMOKINES AND COMMON VARIABLE IMMUNODEFICIENCY; POSSIBLE CONTRIBUTION OF THE FRACTALKINE SYSTEM (CX3CL1/CX3CR1) TO CHRONIC INFLAMMATION

The chemokine fractalkine (CX3CL1) and its receptor CX3CR1 is suggested to play an important role in the pathogenesis of several inflammatory disorders. We hypothesized that enhanced CX3CL1/CX3CR1 interaction could be involved in the chronic inflammation characterising subgroups of CVID. Circulating levels of CX3CL1 were correlated to clinical features in CVID patients and compared with healthy controls. T cells from CVID patients and controls were analysed for expression of CX3CR1 by means of real time RT-PCR and flowcytometry. Peripheral blood mononuclear cells from patients and controls were stimulated with CX3CL1. The main findings were:

- CVID patients were characterized by raised plasma levels of CX3CLl and enhanced expression of its corresponding receptor CX3CR1 on $CD4^+$ and $CD8^+$ T cells, including both $CD45RA^+$ and $CD45RA^-$ subsets.
- CX3CR1 expression was particularly enhanced in patients characterized by chronic inflammation in vivo.
- High expression of CX3CR1 in CVID patients was accompanied by enhanced chemotactic, adhesive, and other inflammatory cell responses to stimulation with CX3CL1.

Conclusion; We demonstrate increased levels of CX3CL1 and CX3CR1 in CVID and suggest that increased CX3CL1/CX3CR1 interaction may contribute to the inflammatory phenotype seen in subgroups of CVID patients.

3.4 PAPER IV; CHEMOKINES AND COMMON VARIABLE IMMUNODEFICIENCY; POSSIBLE CONTRIBUTION OF CCL19, CCL21 AND CCR7 TO IMMUNE DYSREGULATION

The homeostatic chemokines CCL19 and CCL21 and their corresponding receptor CCR7 have recently been associated with modulation of inflammatory and anti-inflammatory responses. Previous reports have demonstrated decreased proportions of $CCR7^+$ T-cells in CVID and in this study we investigated quantitative and functional aspects of CCL19, CCL21 and CCR7 in CVID patients. The main findings were:

- CVID patients had raised serum levels of CCL19 and CCL21 independent of features of chronic inflammation.
- CCL19 and CCR7 was similarly expressed in spleens from CVID patients and controls, while CCL21 was variably down-regulated in spleens from patients.
- T-cell expression of CCR7 was particularly low in patients characterized by chronic inflammation.
- PBMC from CVID patients had attenuated cytokine responses to stimulation with CCL19 and CCL21

Conclusion; We demonstrate enhanced expression of CCL19 and CCL21, and an attenuated cytokine response to stimulation with these chemokines. CCR7, CCL19 and CCL21 are key mediators balancing immunity and tolerance; abnormalities of these mediators might contribute to the profound immune dysregulation seen in CVID.

3.5 PAPER V; LOW NUMBERS OF REGULATORY T CELLS IN COMMON VARIABLE IMMUNODEFICIENCY; ASSOCIATION WITH CHRONIC INFLAMMATION IN VIVO

Abnormalities of $CD4^{+}CD25^{High}FoxP3^{+}$ regulatory T cells (T_{Reg}) have been associated with autoimmune and inflammatory disorders, and we hypothesized that CVID might be characterized by T_{Reg} abnormalities. $CD3^{+}$ cells from patients and controls were analysed for the expression of FoxP3 mRNA by real time RT-PCR. Peripheral blood mononuclear cells from CVID patients and controls were stained for T_{Reg} markers, analysed by flow cytometry and compared to clinical characteristics. The main findings were;

- CVID patients had significantly decreased expression of FoxP3 mRNA and decreased proportions of $CD4^{+}CD25^{High}FoxP3^{+}$ cells compared to controls.
- CVID patients with splenomegaly had lower proportions of T_{Reg} compared than other patients and controls.
- Serum levels of the inflammatory marker neopterin were negatively correlated to the proportions of T_{Reg} within the CVID population, while there was no significant association with bronchiectasis.

Conclusion; We demonstrate decreased proportions of T_{Reg} in CVID patients, particularly in those with signs of an inflammatory phenotype. Decreased proportions of T_{Reg} are suggested to be pathogenetically important in autoimmunity, and our results suggest that T_{Reg} may have a similar role in CVID.

4. DISCUSSION

4.1 METHODOLOGICAL CONSIDERATIONS

Selection of patients

The patients included in the studies of this thesis were recruited from Section of Clinical Immunology and Infectious Diseases at Rikshospitalet University Hospital, Oslo, Norway. Informed consent to blood sampling was obtained from all subjects, and the project has been approved by the regional ethical committee.

Patients included in the study were diagnosed with CVID according to the criteria of the World Health Organization expert group for primary immunodeficiencies, which includes patients with decreased serum levels of IgG, IgA and/or IgM, confirmed in repeated samples with no other specific cause of hypogammaglobulinemia (33). While patients furthermore had onset of symptoms after 2 years of age, in accordance with the stricter definition of CVID as recommended by ESID, data on antibody responses are incomplete. Similar modified criteria have also been used in two recent European studies (36;39) . The estimated onset of symptoms, especially the increased frequency of infections which is the main debut symptom, is often difficult to assess in retrospect. A more precise estimate is possible in the few patients presenting with ITP or hemolytic anemia.

Patients with XLA, Hyper IgM-syndrome and X-linked lymphoproliferative disease were excluded based on current criteria (33). Our centre performs testing for the *Btk*-gene, which is mutated in XLA, in male patients with hypogammaglobulinemia based on clinical suspicion of XLA, i.e. low B-cell count and suspected debut of disease in early childhood.

Interpretation of data from CVID patients merits a note of warning; “variable” is the key word to both clinical and scientific evaluation of these patients, and this means that observed differences between the CVID patients and healthy controls often relates to a subgroup of patients. We have performed B-cell phenotyping according to the EUROCLASS study on 20 patients for manuscript nos 3, 4 and 5 but found no certain association between B-cell phenotype and relevant markers in these studies (39). In manuscript 4 and 5 patients were classified as $CVID_{Hyper}$ if they had splenomegaly (>13 cm on ultrasonographic or CT scan examination) and serum neopterin levels greater than the mean + 4 SD of healthy controls (11.5 nmol/l).

Bronchiectasis were defined based on typical findings on high-resolution computed tomography of the thorax (80;81).The definition of autoimmune disease included idiopathic thrombocytopenic purpura, autoimmune thyroiditis, haemolytic anaemia and autoimmune colitis.

CVID patients are prone to bacterial infections and subsequent antibiotic treatment which may influence measurement of inflammatory markers. However, patients recruited had no clinically apparent infection at the time of sampling and did not receive acute or prophylactic treatment with antibiotics. The anti-inflammatory effect of IVIG is well known, and to minimize this effect in our studies, patients who received IVIG substitution, typically with an interval of 3-4 weeks, were sampled immediately before IVIG administration. Furthermore, data were compared between patients on IVIG and SCIG where concern was raised regarding the effect of IVIG. Auto-immune manifestations in CVID patients sometimes require treatment with anti-inflammatory drugs, most notably corticosteroids. Patients recruited in our study did not receive corticosteroids or other anti-inflammatory treatment.

Known genetic causes of CVID include mutations in ICOS, BAFF-R, CD19 and TACI. While mutations of ICOS, BAFF-R and CD19 has been shown in 9, 1 and 4 CVID patients, respectively, defects in TACI is more frequent and homo- or heterozygote mutations has been described in close to 60 patients so far (77). Our patients have not been tested for known genetic causes of CVID, but the relatively low frequency of mutations suggests any specific finding in our studies would lack statistic power to lead to any reasonable conclusions.

Laboratory methods

Laboratory methods and reagents used in this thesis are thoroughly described in papers I-V, and most will not be further discussed here. However, some of the methods merit general consideration.

All laboratory methods used required blood sampling and preparation from patients and controls. Samples may be exposed to both *ex vivo* activation/apoptosis/necrosis of cells or degradation of proteins, even if standard laboratory procedures as described in the manuscripts are used. Importantly, samples from patients and controls were analyzed at the same laboratory by the same protocol, minimizing any systematic effect of such effects. For flowcytometry of cryopreserved cells, necrotic cells were excluded from analyses by propidium iodide staining.

Protein levels in serum, plasma and cell culture supernatants were analysed by commercially available enzyme-linked immunosorbent assays (ELISA). Although this is a well-proven and much used laboratory method it is worth noting that specificity of reagents varies between manufacturers and also in assays from the same manufacturer. Addressing this, all analyses of a specific protein were run on kits from the same manufacturer. Furthermore, samples for comparison, i.e. samples from patients and controls or samples from unstimulated and stimulated cell cultures, were run on the same microtiter plate.

Expression of extra- and intracellular proteins were assessed by flow cytometry. This is in general a reliable and specific method for quantification of protein expression although it depends on the nature of the protein expression. Proteins expressed in a categorical manner, like the phenotypic markers CD4 and CD8, are generally easier to evaluate than proteins expressed in a continuous manner, like the activation marker CD25. The specificity of antibodies, which can vary between individuals and also between different subsets of cells in the same individual, is at the centre of this discussion. The use of unspecific antibodies of the same isotype as the specific antibody, the so called isotype controls, seeks to assess the degree of unspecific binding in each assay and has been used extensively in our studies here. Unspecific isotypes can either be used to establish a threshold for gating of specifically stained cells, or to calculate the mean fluorescence intensity (MFI) of a specific antibody in a cell population. While gating of positive cells is preferable with categorically expressed markers, the calculation of MFI is preferable with continuously expressed markers. In our studies, both methods were used as appropriate (e.g. gating of positive cells and calculation of MFI in fractalkine and suPAR manuscripts, respectively).

In vitro cell cultures were used to study functional capacity of stimulation with specific proteins on cells from patients and controls. These functional responses were assessed by measurement of secreted cytokines in the supernatant. As for ELISA assays, samples for comparison were run on the same microtiter plate to minimize effect of inter-assay variation. Some proteins have weak stimulatory effect when given alone to cells *in vitro,* while interesting effects are revealed when the protein is given as an adjuvant to cells sub-optimally stimulated with other stimulants. Such co-stimulatory conditions are clearly relevant of the *in vivo* situation where various cells are simultaneously exposed to a wide variety of stimulants. In our studies, we have

used the mitogen phytohemagglutinin (PHA) in sub-optimal doses as determined by preliminary assays on healthy controls (sub-optimal concentration 22.5 ng/ml). Notably, CVID patients have qualitatively and quantitatively different responses to stimulation with mitogens like PHA *in vitro*, notably decreased proliferative response (35). Thus, the sub-optimal dose of PHA in a CVID patient may differ from that of a healthy control, influencing the response to adjuvants. As the cytokine levels in cell culture supernatants from PHA stimulation in CVID patients differed in some degree from that of controls, a confounding effect on the response to adjuvants cannot be excluded. However, titrating the sub-optimal PHA response for both patients and controls to apply different doses of PHA to these two groups would cause a problematic methodological inconsistency. Future studies should also examine the effect of other co-stimulants with relevance to CVID such as bacterial products and other cytokines.

Statistical methods

In line with the heterogeneous nature of CVID, most of the data analyzed in this thesis do not have a normal distribution and non-parametric methods of statistics have therefore been used. Non-parametric statistics are less influenced by outliers as found in heterogeneous data, but the use of ranks instead of absolute values and the impact of *n* analyzed on the p-value are a limitation. When reporting non-parametric data, it is customary to report median and a non-parametric measure of variability, such as 25^{th} to 75^{th} percentiles. However, to improve readability in the highly condensed format required by medical journals, data are presented as mean ± SEM in some figures.

4.2 DISCUSSION OF RESULTS

4.2.1 Innate immune mechanisms in CVID

Innate immunity can contribute to CVID pathogenetically through inept innate responses and subsequent inappropriate antigen-presentation, as suggested through immaturity of dendritic cells in CVID. The failure of the adaptive immune system in CVID can also make patients reliant on phylogenetically older and more primitive

immune responses, putting a strain on these and theoretically lead to either failure or compensatory enhancement of these mechanisms.

MBL deficiency has been associated with respiratory infections in children although later studies have either have shown a weaker association than previously reported or no association at all (14;15;82-84). In an adult population low MBL levels do not seem to increase mortality or the occurrence of infectious disease, although conflicting data exist also here (16;85). However, low MBL levels have been associated with a poor prognosis in cystic fibrosis and also in immunocompromised hosts (86-88). The widespread existence of MBL deficiency suggest that MBL is of minor importance from an evolutionary point of view, and Turner et al. have proposed that pathology arising from MBL deficiency may require one or more co-existing immune deficits (89).

The complement system, including the MBL pathway, has an important role in the innate immune response and dysregulation of this system may contribute to both susceptibility to infections and increased frequency of autoimmune and inflammatory manifestations in CVID. Indeed, Andersen et al. reported an association between structural mutations of the *mbl2* gene and increased frequency of severe respiratory tract infections in CVID, and others have reported an association between low producing MBL alleles and earlier age of disease onset and autoimmunity in CVID (90;91).

The study presented in paper I did not show increased prevalence of low MBL levels or lectin pathway deficiency in CVID patients compared to controls. This is consistent with the finding of Mullighan et al. in a study of 160 CVID patients in the UK (91). However, individuals with lectin pathway deficiency have a putative abnormal MBL synthesis and this may mask relevant differences in functional capacity of the lectin pathway and serum concentration of MBL between patients and controls with a normal synthesis of the protein (92;93). Thus, excluding subjects with lectin pathway deficiency, focusing on patients and controls with a normal MBL synthesis, functional capacity of the lectin pathway and MBL levels were significantly higher among CVID patients than controls. MBL has been considered a weak acute phase reactant, but although CRP levels were also raised among CVID patients, they did not correlate to MBL levels, suggesting at least partly different mechanisms of regulation. Moreover, the patients in the present study had no obvious acute infection at the time of sampling and the stable MBL levels found during longitudinal testing

makes an elevation caused by intercurrent acute inflammation improbable. Elevated levels of MBL have also been reported in studies of chronic diseases such as cystic fibrosis, HIV infection and rheumatic heart disease (94-96). Little is known of factors regulating MBL beyond polymorphisms in the promoter regions and mutations in the MBL gene, and the reason for elevated levels of MBL and the possible clinical correlates in these chronic diseases remain largely unexplained.

The major finding presented in paper I was a strong inverse correlation between MBL levels as well as functional capacity of the lectin pathway and the frequency of lower respiratory tract infection and bronchiectasis in the CVID group. In the before mentioned study by Andersen et al there was an association between structural mutations of the *mbl2* gene and increased frequency of severe respiratory tract infections in CVID (90). In our study we extended these findings in several ways. While most studies, including the study by Andersen et al, have focused on MBL deficiency as defined by the presence of mutations in the MBL gene, we show a more continuous association between serum concentrations of MBL and lower respiratory tract infection and bronchiectasis. In fact, patients with MBL levels below 500 ng/ml had increased frequency of lower respiratory tract infection and bronchiectasis, while patients with MBL levels above 4000 ng/ml had reduced frequency of these respiratory complications. Thus, it seems that while low MBL levels may predispose to lower respiratory tract infection and bronchiectasis in CVID, high MBL levels may protect against these complications in CVID. MBL can bind to a variety of bacteria and other microbes as well as their potentially toxic components, neutralizing them and/or opsonizing them by activating complement through the lectin pathway (97). It has furthermore been isolated in physiologically active levels in alveolar fluid from children with lower respiratory tract infection, suggesting that MBL is operating in the pulmonary microenvironment, giving some protection against the development of respiratory tract complications including bronchiectasis (98). Even though no data exist comparing MBL levels in alveolar fluid to MBL levels in the circulation, a mutated MBL gene would quite possibly lead to lower alveolar levels of MBL. Thus, MBL levels in CVID patients could contribute to airway complications, supporting, together with other studies, the principle proposed by Turner. Our finding of correlation between MBL levels and pulmonary complications in CVID has since been confirmed in another study (99).

There is a close correlation between the different MBL alleles and serum concentrations of MBL, but there is also a wide range of serum concentrations within each allele and a significant overlap of serum concentrations between these alleles (11-13). A mapping of MBL alleles could potentially help to identify MBL deficient subjects and was done in the study of Litzman showing increased frequency of respiratory complications to both genetically and biochemically assessed MBL-deficiency (99). Importantly, genetic studies will not discriminate between normal MBL allele individuals with intermediate and high serum levels of MBL. Our study suggests that such discrimination may be clinically relevant as the various effects of MBL may be dose-dependent.

Our findings indicating a protective effect of particularly high MBL levels may also suggest a therapeutic potential for MBL infusions as tested in small experimental studies (100). Furthermore, the identification of MBL in alveolar fluid in lower respiratory tract infection suggest the therapeutic potential of local, rather than systemic, administration of MBL in lower RTI, perhaps even in supra-physiological doses.

There are a few reports, mostly case studies, on complement deficiencies in CVID and the possible contribution of defects in the classical and alternative pathways has been uncertain (56;57). However, in our systematic study of the complement system in CVID we did not find any significant deficiencies of the classical or alternative pathways. We did, however, find signs of complement activation and this will be discussed in the next chapter.

As evident by the vascular changes in acute inflammation, circulation and circulatory components are interwoven with human immune responses. The uPA system has an ambiguous role in infectious diseases where it may be involved in both harmful and beneficial inflammatory mechanisms during host response. In murine knock-out models loss of uPA is associated with low recruitment of T cells, granulocytes and macrophages to inflammatory tissue, and enhanced risk of bacterial infections (101-105). Furthermore, the uPA system is an activator of neutrophil granulocytes, enabling microbe killing by superoxide mechanisms, and uPA itself is shown to act as an endogenous antibiotic (8;106;107). As for uPA, uPAR has been shown to be involved in various inflammatory processes in murine knock-out models, most notably in the defence against bacterial infections (108;109). In bacterial infections, murine models support a beneficial role of uPAR through recruitment of

neutrophil granulocytes to inflamed tissue, and while this is generally considered protective it has been suggested to be harmful during meningitis (108-110). In HIV infections, high levels of suPAR are associated with a poor prognosis (111). The role of the uPA system in these innate immune responses is of particular interest in CVID where, as we have suggested through paper I, the compromised adaptive immune response potentially makes patients reliant on innate mechanisms of immune defence. Thus, the innate properties of an activated uPA system may be beneficial in acute and chronic bacterial infections in CVID. In the study presented in paper II we found increased circulating levels of suPAR and uPA, as well as increased monocyte expression of uPAR, suggesting an activation of the uPA system in CVID. We saw no correlation of suPAR to features associated with recurrent or chronic bacterial infections, making the potential link between suPAR and innate infectious responses uncertain.

Raised levels of MBL and the activation of the uPA system may both contribute to increased innate protection in patients with a failing adaptive immune response. Although much is unknown about the specific regulation of both MBL and the uPA-system, the potential consequence of their up-regulation makes it tempting to consider their up-regulation as representing some form of compensatory mechanism.

4.2.2 Inflammatory mechanisms in CVID

The inflammatory manifestations of CVID are clinically heterogeneous. All patients are subject to inflammation during intercurrent infections or autoimmune disease but some patients display immune activation even when no active infection or autoimmune disease has been detected suggesting an underlying inflammatory phenotype (42;45;55). The constitutive nature of this inflammation in CVID is supported by the contrast to patients with XLA, which despite their hypogammaglobulinemia and intercurrent infections fail to exhibit these inflammatory manifestations. In investigating these features of CVID, the exclusion of patients with current infections or autoimmune disease requiring anti-inflammatory treatment, like active ITP or arthritis, is of importance. The manifestations of this inflammatory phenotype are persistent and thus chronic, but of a different nature than chronic inflammation seen in a patient with e.g. a chronically infected pulmonary focus. We have used the term chronic inflammation *in vivo* to describe this phenotype

and it shares some characteristics with the para-inflammation suggested by Medzhitov; there is no classic chronic inflammatory focus but rather a systemic and persistent activation of the immune system (5). The characteristics of this inflammatory phenotype will be discussed here as well as in the next chapter of regulation of inflammation in CVID.

Immunological characteristics of this inflammatory phenotype includes enhanced levels of markers consistent with persistent immune activation, in particular markers associated with monocyte activation (43;45;55). Furthermore, several studies have pointed towards a T_H1 favored immune activation in CVID, based on increased serum levels of IL-12, as well as enhancement of the IL-12/IFN-γ cytokine loop in monocytes and T cells (47;49;50). CVID is also characterized by relatively high levels of $CCR7^-$ and $CD4^+CD45RA^-$ T cells in peripheral blood, corresponding to a shift from a naïve lymph-node homing phenotype to an effector/memory tissue-homing phenotype of T cells (62;64-66). Clinically, these patients are characterized by lymphoid hyperplasia, typically splenomegaly, and high frequency of autoimmune disease, in particular ITP. Thus, identifying patients with chronic inflammation *in vivo* we have classified patients based on serum levels of neopterin and presence of splenomegaly into the $CVID_{Hyper}$ group as has been accounted for previously. I will now consider the contribution of factors studied in this thesis on this inflammatory phenotype.

Contribution of the complement system

The complement system plays a central role in the host defence against microbes but enhanced complement activation may also induce tissue damage and inflammation. Accordingly, a dysregulated complement system has been associated both with increased susceptibility to infections and autoimmune diseases (9;10). In CVID, low producing MBL alleles has been associated with autoimmunity but in paper I we found no such association between low serum levels of MBL and autoimmunity within the CVID group (91). Interestingly, CVID patients had increased functional capacity in both the classical and alternative pathways compared to controls, with increased levels of complement split products (i.e., C3a, C4a and C5a).This complement activation, and in particular the release of inflammatory mediators such as C5a, could potentially contribute to inflammatory and autoimmune manifestations characterizing CVID. Indeed, we found an association between high levels of factor B

and splenomegaly, between high levels of factor B, C1q and C3 and granulomatous disease, and between high levels of C3a and idiopathic thrombocytopenia. These findings suggest that enhanced complement activation is another inflammatory characteristic of CVID patients.

Contribution of the uPA system

Enhanced uPA activation has been seen in inflammatory disorders such as rheumatoid arthritis, atherosclerosis and asthma (112-114). In humans, high levels of components of the uPA system, which include uPA, uPAR and suPAR, have been found in malignant, inflammatory and infectious diseases (115-119). In paper II we show increased plasma levels of suPAR in all CVID patients, with particularly high levels in subgroups with an inflammatory phenotype characterised by thrombocytopenia and splenomegaly. Levels of uPA followed the same pattern with significant enhancement in patients with splenomegaly and thrombocytopenia. Within the CVID population levels of both suPAR and uPA correlated to neopterin, which have been shown to covariate with suPAR in other studies(120;121). Moreover, monocytes from CVID patients had enhanced cellular expression of uPAR, further supporting a link between increased activity in the uPA system and monocyte activation in these patients. Indeed, activated monocytes are a probable candidate for suPAR production in CVID, with a possible contribution of enhanced uPA-mediated proteolytic activity in subgroups of patients (122;123). While our study found a strong association to monocyte activation we also found a down-regulation of uPAR in T cells from CVID patients. Lymphocytes are generally a poor reservoir for uPAR even if increased expression is seen on activation of T cells, and the observed down-regulation might reflect the phenotypic shift of T cells previously reported in CVID, including an altered CD4/CD8 cell ratio and low levels of naïve $CD4^+$ cells (124;125). Nevertheless, *in vitro* studies and studies in animal models suggest a role for uPAR in T-cell activation promoting cell proliferation and release of cytokines, including T-cell derived B-cell growth factors (126). The decreased expression of uPAR on T cells from these patients may potentially contribute to impaired T-cell function seen in subgroups of CVID patients, but the findings in paper II primarily supports a role for the uPA system in monocyte activation in CVID.

Contribution of the fractalkine system

The high expression of chemokines is a characteristic feature of inflammation, and importantly their chemotactic effects are supplemented by their inflammatory effects, meaning chemokines will both direct and activate cells. The chemokine fractalkine, CX3CL1, and its receptor, CX3CR1, are associated with the pathogenesis of several inflammatory disorders such as glomerulonephritis, rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis and HIV infection (127-135). We have previously observed that the gene for CX3CR1 is up-regulated in T cells in a group of CVID patients characterized by low T-cell proliferation *in vitro*, and in paper III we investigated more properties of the fractalkine system in CVID (64). Elevated levels of CX3CL1 in serum have been observed in various inflammatory conditions, and it has been suggested to reflect enhanced localized release from an inflamed and activated endothelium (130;131;133;134;136). We found raised levels of CX3CL1 in all CVID patients, while there was no elevation of vWF, a known marker of endothelial activity. The differential secretion of CX3CL1 and vWF from endothelium is documented *in vitro*, where CX3CL1 expression is induced by IFN-γ, while expression of vWF is attenuated (137-139). Notably, increased IFN-γ activation has been reported in CVID suggesting that a similar mechanism of selective activation of CX3CL1 may occur in CVID *in vivo.* Alternatively, CX3CL1 may be produced by extravascular cells as the chemokine is constitutively expressed in various tissues, among them skeletal muscle, colon and lung, and can be induced in several cell types including smooth muscle cells, dendritic cells and intestinal epithelium (127;140-142).

We confirmed our previous microarray experiments through showing increased CX3CR1 expression on T cells from CVID patients by means of flow cytometry and PCR, and interestingly, the receptor was most markedly expressed on T cells from the $CVID_{Hyper}$ subgroup. Normally, the receptor CX3CR1 is expressed on cells from the hematopoetic lineage, most notably in monocytes and cytotoxic lymphocytes such as NK-cells, γδ T cells and CD8+ effector cells, but also to some extent in $CD4^+CD45RA^-$ effector memory cells (129;143). While we did find relatively high levels of both $CD8^+$ cells and $CD4^+CD45RA^-$ cells in the CVID population, this could not account for the increased expression of CX3CR1 in the $CVID_{Hyper}$ group as this was observed in $CD4^+$ and $CD8^+$ cells, as well as $CD45RA^+$ and $CD45RA^-$ cells. Thus, the increased expression of CX3CR1 in T cells from

$CVID_{Hyper}$ patients does not merely reflect a phenotypic shift of T cells in peripheral blood of these patients. Importantly, it seems that CX3CL1 induce inflammatory responses in PBMC from $CVID_{Hyper}$ patients, suggesting that the increased expression of CX3CR1 in cells from these patients may affect their functional responses. Thus, increased serum levels of CX3CL1 as well as enhanced T-cell expression of CX3CR1 in $CVID_{Hyper}$ patients may contribute to the inflammatory phenotype seen in these patients. In contrast, while CX3CR1 is involved in monocyte survival and function, we found no differential regulation of the receptor in monocytes from CVID patients (144).

Previous studies have shown pro-inflammatory effects of CX3CL1 associated with a T_H1 response (145;146). As CX3CR1 has been localized in polarized T_H1 cells and CX3CL1 expression in endothelial cells is induced by CD40L and IFN-γ, the CX3CL1/CX3CR1 system has been suggested to be part of an amplification circuit of a T_H1 response (146;147). The upregulation of the CX3CL1/CX3CR1 system demonstrated in paper III may therefore also reflect an amplification and polarization of the previously suggested T_H1 skewed response in CVID (47).

We have previously shown extensive T-cell abnormalities in CVID, ranging from increased cytotoxic activity to alteration of adhesion processes and our findings presented in paper III supports the role of T-cell pathology in CVID (64;148-150).

Contribution of CCL19, CCL21 and CCR7

While most chemokines have been linked to inflammatory processes in peripheral tissue, the homeostatic chemokines CCL19 and CCL21 and their corresponding receptor CCR7 have traditionally been associated with development and maintenance of secondary lymphoid organs, as well as the entry of lymphocytes and dendritic cells to secondary lymphoid tissue (151;152). Recently, however, reports have pointed to a broader role for these homeostatic chemokines, including modulation of inflammatory and anti-inflammatory responses in peripheral tissue. An imbalanced regulation of CCL19 and CCL21has been suggested to be involved in the pathogenesis of various inflammatory disorders including rheumatoid arthritis, inflammatory bowel diseases and atherosclerosis (153-157). In paper IV we extend these findings by showing raised serum levels of CCL19 and CCL21 in CVID patients as compared with controls. While CCL19 and CCL21 are mainly expressed in lymphoid tissue, circulating levels of CCL19 and CCL21 possibly also reflect expression in peripheral

inflamed tissue. CVID patients are prone to both recurrent and chronic bacterial infections, providing inflammatory foci that might contribute to levels of CCL19 and CCL21. Bronchiectasis are frequently associated with bacterial infection in CVID and while both patients with and without bronchiectasis had raised levels of CCL19 compared to controls this was most notable in patients with this complication. It is therefore possible that the infections seen in CVID patients with bronchiectasis contribute to the high levels of CCL19 seen in this subgroup of patients. The presence of splenomegaly, contributing to increased lymphoid mass in the $CVID_{Hyper}$ group, was not correlated to serum levels of CCL19 and CCL21. Furthermore, there was a similar expression of CCL19 in spleen tissue from both controls and CVID patients, while CCL21 was variably down-regulated in spleens from patients, making spleen a less likely source for the elevation of these chemokines in patients. Monocytes from the $CVID_{Hyper}$ group tended towards enhanced expression of CCL19, and a possible contribution from monocytes to serum levels of CCL19 in this subgroup cannot be excluded. Thus, even if the raised serum levels of CCL19 might be related to both inflammatory cells associated with bronchiectasis and monocyte activation, the serum levels of CCL19 and CCL 21 seem elevated independently of features of chronic inflammation in CVID.

As commented upon, we and others have previously shown the down-regulation of CCR7 on T cells in groups of CVID patients (64-66). In paper IV we supplement these findings by showing particularly low proportions of $CD3^{+}CCR7^{+}$ in the $CVID_{Hyper}$ group, suggesting that this characteristic is another inflammatory feature of this subgroup. Interestingly, while expression of CCR7 is markedly affected in the $CVID_{Hyper}$ group, the levels of CCL19 and CCL21 are not and moreover, there is no correlation between levels of CCL19 and CCL21 and T-cell expression of CCR7. Thus, even if CCL19 has been shown to modify cellular CCR7 expression, our study suggests that expression of CCR7 on circulating T cells is regulated at least partly independent of its ligands in CVID (158).

Stimulation of $CCR7^{+}$ cells with CCL19 and CCL21 in various settings has shown important modulation of cell responses by these chemokines, but in contrast to the modulatory effects of CCL19 and CCL21 seen in PBMC from healthy controls, cells from CVID patients gave no significant response to CCL19 and CCL21 stimulation (155;159;160). The results presented in paper IV supports a constitutive

enhancement of CCL19 and CCL21 in CVID, while CCR7 seems related to inflammatory features.

Concerning lymphoid hyperplasia and granulomatous disease

In paper IV, we present histological characteristics of splenomegaly in nine CVID patients, and notably splenomegaly in CVID is histologically heterogeneous. Some patients have markedly increased white pulp consistent with an increase of lymphoid cells in the spleen while others have normal or even small white pulp. In paper III-IV we demonstrate increased levels of chemokines normally expressed in secondary lymphoid tissue; CX3CL1, CCL19 and CCL21 but only CX3CL1 was associated with lymphoid hyperplasia. Furthermore, T cells from the $CVID_{Hyper}$ group showed significantly enhanced chemotactic and adhesive properties to stimulation with CX3CL1. As CX3CL1 is strongly expressed in high endothelial venules of reactive hyperplastic lymph nodes it is tempting to suggest that the enhanced CX3CR1 expression on T cells might be involved in an abnormal pattern of lymphocyte migration in CVID (161).

Serum levels of CCL19 and CCL21 were expressed independently of lymphoid hyperplasia but their receptor, CCR7, was not. In contrast to the diminished CCR7 expression on circulating T cells in CVID, spleens from CVID patients showed a similar expression of CCR7 as compared to controls, suggesting the down-regulation of CCR7 in CVID does not affect all lymphoid compartments.

4.2.3 Regulatory immune mechanisms in CVID

Regulatory immune mechanisms are closely linked to autoimmunity. The apparent paradox of increased frequency of both infections and autoimmunity in CVID defy a view of infections and autoimmunity as examples of merely attenuated and exaggerated immune responses, respectively. Several mechanisms have been suggested to cause autoimmunity in CVID, including defective clearing of external antigens, genetic predisposition, immune dysregulation and breakdown in mechanisms of tolerance (162). However, the growing understanding of tolerance to innocuous antigens as an active process requiring presence and function of specially designated cells, mimicking the response to pathological antigens, has clarified a possible link between autoimmunity and infections in CVID (24).

The concept of tolerance was introduced previously, and while defective central tolerance, e.g. failure of apoptosis of autoreactive lymphocytes, might contribute to autoimmunity in CVID this has been scarcely commented upon. Peripheral tolerance can be mediated by several subgroups of T cells, and in particular the role of T_{Reg} in conditions such as transplant rejection reactions, cancer, autoimmunity, and chronic infectious diseases has been a subject of active investigation (163-167). In paper V we investigated whether CVID, with its high frequency of autoimmunity and signs of chronic inflammation, might be characterized by T_{Reg} abnormalities.

We show that CVID patients have decreased proportions of T_{Reg} as compared to controls. The decreased proportions of these $CD4^{+}CD25^{High}FoxP3^{+}$ cells in CVID is further supported by low expression of FoxP3 mRNA in $CD3^{+}$ cells in the same patients. Interestingly, we find the lowest proportions of T_{Reg} in CVID patients with splenomegaly and a negative correlation between levels of neopterin and proportions of T_{Reg}. Thus, we find a link between decrease of T_{Reg} and CVID patients characterized by chronic inflammation *in vivo.* This link raises interesting questions regarding both homeostasis and function of T_{Reg}. The immediate reason for the low numbers of T_{Reg} in CVID patients with splenomegaly is unclear; while low numbers of circulatory $CD4^{+}CD25^{High}$ cells have been observed in some other diseases, even without splenomegaly, this is less well documented for $CD4^{+}CD25^{High}FoxP3^{+}$ cells (164;168;169). The homeostasis of T_{Reg} is controlled by several factors, among them the differentiation of T_{Reg} in the thymus, induction and/or expansion of T_{Reg} in the periphery, half-life of T_{Reg} in the circulation and possible redistribution of T_{Reg} to extra-vascular tissue (170). We can only speculate which factors contribute to our finding but one possibility is that the observed maturation defects of effector T cells in CVID also applies to the development of T_{Reg}. The link between low numbers of T_{Reg} and chronic inflammation can also be seen from a more functional perspective. In acute inflammation it has been suggested that feed-back mechanisms cause a relative decrease in T_{Reg} early in the process to allow for an effective immune response, followed by an increase in T_{Reg} to reduce the response when the antigen is cleared (21). In chronic inflammation, low numbers of T_{Reg} could reflect dysfunctional feedback mechanisms, failed clearance of an offending antigen or a more constitutive defect of T_{Reg} as seen in the IPEX syndrome (24).

T_{Reg} execute their suppressive role through different mechanisms, including

the secretion of IL-10 and TGF-β. We have previously reported decreased secretion of IL-10 in T-cell cultures from CVID patients, and interestingly we found a correlation between secreted IL-10 levels in T-cell cultures and T_{Reg} numbers in CVID, supporting the link between IL-10 and T_{Reg} (70). Recent studies have further suggested that a balanced interaction between CCL19, CCL21 and CCR7 is necessary not only for the optimal initiation of protective immunity against various microorganisms, but also for the induction of peripheral tolerance and the regulation of the immune response by T_{Reg} (151;171;172). It is tempting to hypothesize that the inability of CCL19 and CCL21 to induce the release of several important immunoregulatory cytokines in PBMC from CVID patients, as presented in paper IV, could be involved in the defective regulation of the immune response in CVID. CCL19/CCL21 deficiency has been associated with both a delayed and exaggerated response to antigens in a murine model, a picture mirroring the increased frequency of infections and autoimmunity seen in CVID (171).

The results presented in paper V show a role for T_{Reg} in CVID pathology, in particular in patients with chronic inflammation *in vivo,* suggesting that this immunodeficiency is characterised not only by inept adaptive responses and chronic inflammation, but also a profound dysregulation of cellular responses. The potential of T_{Reg} associated anti-inflammatory therapy might thus also apply to CVID.

5. CONCLUSION

CVID represents an immunodeficiency enabling us to understand not only the characteristics of a defect immune system but also important aspects of the functional immune system. In the studies of this thesis we present novel insights into innate, inflammatory and regulatory immunological features of CVID that contribute to the multi-faceted pathology of this disease. Notably, we suggest the possible detrimental contribution of MBL deficiency to respiratory complications in patients already deficient of an adequate adaptive immune response. We show an inappropriate activation of the suPAR and fractalkine systems associated with a subgroup of CVID patients characterised by an inflammatory phenotype. We find constitutively enhanced levels of the chemokines CCL19 and CCL21 possibly contributing to the immune dysregulation in CVID. Last, we demonstrate decreased proportions of T_{Reg} in CVID patients, particularly in those with signs of an inflammatory phenotype, further suggesting a profound perturbation of the immune system in CVID.

The mainstay treatment of CVID today is substitution of IgG through intravenous or subcutaneous administration. This leads to drastic improvement of symptoms in many patients but some still suffer from particularly respiratory infections, while others present with autoimmune disease not conveniently treated in immunodeficient patients. The studies presented in this thesis add to the knowledge that may be needed to address these clinical problems in CVID, while also shedding light on processes of importance in other immunodeficiencies and inflammatory disease.

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